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VARIOUS METHODS FOR DETERMINING THE TRYPANOCIDAL ACTIVITY OF SUBSTANCES IN VITRO AND THEIR RELATION TO THE CHEMOTHERAPY OF EXPERIMENTAL TRYPANOSOMIASIS *

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During the course of chemotherapeutic studies, compounds possessing a well-defined parasitropic effect may be prepared which prove so highly organotropic or toxic for the test animal that the minute doses tolerated fail to exert an appreciable influence on the parasites. The mercurials, which are known to exert a powerful bactericidal effect in vitro, are substances of this class, being so highly toxic that doses greater than 0.0004 gm. per 100 gm. of white rat are apt to prove fatal and yet are too small materially to influence the course of experimental trypanosomiasis.

In order, therefore, to avoid the mistake of discarding new compounds possessing a high degree of trypanocidal activity because they are highly toxic for experimental animals, a study of various methods of determining the trypanocidal activity of a drug independent to some extent of its toxicity for the body cells of the host, was undertaken. In the event of the preparation of a new compound that is possessed of a high degree of parasitropism, but is also highly toxic, the efforts of the experimental chemotherapeutist are devoted to lowering the toxicity to a sufficient extent to permit of the administration of the drug to living animals.

With the purpose, then, of devising a technic for determining the parasiticidal activity of a chemical independent of its toxicity for body cells, we have studied various methods of determining the trypanocidal activity of substances in vitro. There is considerable evidence at hand that substances exhibiting a marked parasiticidal effect in vitro are likely to act in a similar manner in vivo, provided the toxicity is sufficiently low to permit of the administration of fair-sized doses; this is true to a certain extent of arsenobenzol, as will be shown

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later in this communication, and has been found true of ethylhydrocuprein hydrochlorid and other quinin derivatives in the treatment of experimental pneumococcus infections.[1]

Since the cultivation of pathogenic trypanosomes, as T. equiperdum and T. brucei, on artificial media yields irregular results, our methods were designed to determine the influence of a drug upon trypanosomes in vitro by intraperitoneal injection of drug and trypanosome into white rats—such influence to be observed in loss of motility and structural changes, determined by direct microscopic examination, and more particularly in whether or not the trypanosomes were killed.

Several of these methods are similar to those devised by one of us (Kolmer[2]) for determining the bactericidal action of substances in vitro with reference to the chemotherapy of bacterial infections. The microscopic method described herein is essentially similar to that described by Kolmer and Smith[3] in a study of the trypanocidal activity of emetin in vitro.

MICROSCOPIC METHOD

Technic.—A series of dilutions of the chemical in 0.85% sodium-chlorid or Ringer's solution and twice as strong as those desired for action on the trypanosomes, is prepared in amounts of 0.5 c.c. and placed in small sterile test tubes in a suitable rack in a pan of water at 37-40 C.

A seed rat carrying the trypanosomes for the tests is selected whose blood as examined microscopically with a 1/6 objective and No. 4 eyepiece (Leitz) by placing a drop from the tail upon a cover glass and suspending it over a concave slide, shows a large number of active trypanosomes. One cubic centimeter of a 1% solution of sodium citrate in normal salt or Ringer's solution is placed in a test tube and warmed; sufficient blood is then secured from the heart or tail of the seed rat to give an emulsion of trypanosomes which, when examined as described above, will show at least 10 trypanosomes in each field and particularly at the margin of the drop. The sodium citrate is added to prevent coagulation of the blood, but more than a 2% solution is not advisable. This blood trypanosome emulsion is kept warm by placing the tube in the water bath.

Slides having two or more concavities are prepared and ringed with vaselin. With a 3-mm. platinum loop a drop of a dilution of the chemical is placed in the center of a cover glass; the wire is heated and cooled and a drop of the blood emulsion added and mixed in exactly the same manner as in preparing a microscopic agglutination test. The cover glass is then adjusted to a concavity in the slide and carefully sealed with vaselin. The dilution of chemical now acting upon the trypanosomes is doubled. The time when blood and chemical are mixed is carefully noted, and the slide placed on a warm stage of the microscope or in a small oven heated to 37-40 C. placed alongside of the microscope.

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[1] This report is to be published later.

[2] This report is to be published later.

[3] Jour. Infect. Dis., 1916, 18, p. 247.

A number of preparations are made as rapidly as possible with the aid of an assistant, including at least 2 controls prepared by mixing a drop of trypanosome emulsion with a drop of warm normal salt or Ringer's solution.

It is advisable to keep the solutions of chemical, the trypanosome emulsions, and the finished slides at body temperature in order to avoid the effect of chilling on the trypanosomes.

At definite intervals, ranging, for example, from 5 or 10 minutes to an hour or longer from the time of exposure of the trypanosomes to the chemical the slides are examined microscopically and compared with the controls.

As a general rule, the controls show active trypanosomes for at least 1 to 2 hours or longer; after this time the movements may become sluggish or cease entirely in a few of the parasites. For the purpose of avoiding error it is our custom to terminate the experiment at the end of 1 hour.

In case the chemical under study exerts an influence on the trypanosomes, the parasites first lose their to and fro movements and remain in one position with constant vibratile movement. Later the latter movement becomes more and more sluggish, finally ceasing altogether, and certain structural changes occur. These effects are best determined by inspecting the trypanosomes at the margin of the drop; before motility is regarded as completely destroyed, a number of fields should be carefully inspected.

With practice and assistance a large number of preparations may be made in a short time. It is our custom to permit at least one-half minute for the preparation of each cover glass. As a general rule it is advisable to examine a preparation within 10 minutes of the time when chemical and trypanosomes are mixed; strong dilutions of a trypanocidal substance, as arsenobenzol, produce an effect within a few minutes.

The results of a few experiments with various substances conducted after this method are shown in Tables 1, 2, and 3.

Of particular interest are the effects noted with arsenobenzol, which has been claimed to be relatively inert in vitro, and with bichlorid of mercury, which is so toxic that a sufficient dose to influence trypanosomiasis in the living animal cannot be given.

This method is open, however, to some degree of error. The results of experiments with the same substance at different times with the same strain of trypanosomes have been found to vary, hence the results should be interpreted as showing approximately the relative trypanocidal activity of different substances judged solely by their influence on the motility and appearance of the parasites; loss of motility, however, is not necessarily an indication of the death of a parasite.

THE IN-VITRO-VIVO CENTRIFUGE METHOD

In this method the trypanosomes are exposed for a certain interval to a definite dilution of chemical, are then removed and washed by centrifugation, and injected into the peritoneal cavities of white rats to determine whether or not their destruction has been brought about. The test thus becomes a combined in-vitro-vivo test.

TABLE 1

Trypanocidal Activity of Emetin

Dilutions	Minutes of Time Required to Kill all Trypanosomes	
	T. Lewisi	T. Equiperdum
1 : 100	3 to 10	17 to 55
1 : 200	10	13 to 32
1 : 400	12	11 to 19
1 : 800	14	21 to 30
1 : 1600	15 to 18	30 to 41
1 : 3200	20 to 40	40 to 50
1 : 6400	25 to 45	60 to 72
1 : 12800	30 to 70	100 to 130
1 : 25600	60 to 90	125 to 140
1 : 51200	70 to 120	150 to 190
1 : 102400	80 to 140	

TABLE 2

Microscopic Trypanocidal Test with Bichlorid of Mercury and T. Equiperdum

Dilutions	Loss of Motility After Exposure, in Minutes								
	8	12	16	20	24	30	40	50	60
1 : 100	—								
1 : 150	—								
1 : 200	—								
1 : 300	—								
1 : 400	—								
1 : 600	—								
1 : 800	—								
1 : 1200	—								
1 : 1600	—								
1 : 2400	—								
1 : 4800	+	—							
1 : 6400	+	—							
1 : 12800	+	+	+	+	—				
1 : 25600	+	+	+	+	—				
1 : 50000	+	+	+	+	+	—			
1 : 100000	+	+	+	+	+	+	+	+	+
1 : 200000	+	+	+	+	+	+	+	+	+
Controls	+	+	+	+	+	+	+	+	+

+ = motility; — = loss of motility.

TABLE 3

Microscopic Trypanocidal Test with Arsenobenzol and T. Equiperdum

Dilutions	Loss of Motility After Exposure, in Minutes								
	8	12	16	20	24	30	40	50	60
1 : 100	—								
1 : 150	+	—	—						
1 : 200	+	+	—						
1 : 300	+	+	+	—					
1 : 400	+	+	+	—					
1 : 600	+	+	+	—					
1 : 800	+	+	+	+	—				
1 : 1200	+	+	+	+	—				
1 : 1600	+	+	+	+	+	—			
1 : 2400	+	+	+	+	+	—			
1 : 4800	+	+	+	+	+	—			
1 : 6400	+	+	+	+	+	—			
1 : 12800	+	+	+	+	+	+	—		
1 : 25600	+	+	+	+	+	+	+	+	—
1 : 50000	+	+	+	+	+	+	+	+	+
1 : 100000	+	+	+	+	+	+	+	+	+
1 : 200000	+	+	+	+	+	+	+	+	+
Controls	+	+	+	+	+	+	+	+	+

+ = motility; — = loss of motility.

A strain of trypanosomes which is pathogenic for white rats should be used, such as T. equiperdum or T. brucei; the former, which usually kills the host in from 4 to 7 days after inoculation, has been found uniformly satisfactory.

Technic.—A seed rat is selected whose blood, as examined in a drop from the tail, shows the presence of a large number of trypanosomes. If it is desired to expose approximately known numbers of trypanosomes to definite dilutions and amounts of chemical, as is advisable in comparative tests, the trypanosomes may be counted by the method of Kolmer[4] and the approximate number per cubic centimeter of blood determined. Experience has shown us that it is advisable to work with an emulsion containing at least 100,000,000,000 trypanosomes per cubic centimeter.

Blood is drawn from the heart or from the vessels in the neck into a measured amount of a warm 1% solution of sodium citrate in normal salt solution until the desired dilution is obtained. If an actual count is not made, the blood of a heavily infected rat may be diluted with 3 parts of this solution.

Dilutions of the chemical in warm normal salt solution and in amounts of 0.5 c.c. are prepared in centrifuge tubes and placed in a water bath at 40 C.; to each tube and several controls of salt solution alone, is now added 0.5 c.c. of the blood trypanosome emulsion and the tube gently shaken. After an exposure of 15 minutes, 10 or 15 c.c. of warm salt solution are added to each tube to dilute the chemical and wash the trypanosomes, and the tubes are centrifugated at high speed.

To insure uniform success it is imperative to avoid the extreme chilling consequent upon rapid centrifugation. To this end the centrifuge cup should be large and filled with water at 42 C. Even under these circumstances chilling may occur and cause irregular results.

The supernatant fluid is now removed; 1 c.c. of warm salt solution is added to each tube, and the sediment of blood cells and trypanosomes is mixed and injected into the peritoneum of a white rat.

Daily examinations are made of the blood from the tail and the trypanocidal influence of the chemical judged according to the time elapsing before trypanosomes appear in the peripheral blood and the duration of life thereafter as compared with that of the controls.

The results of an experiment conducted with arsenobenzol are shown in Table 4.

In conducting this test it is necessary to use large numbers of trypanosomes and to avoid chilling as much as possible. The rats should be kept under observation for at least 2 weeks before being considered sterile, as trypanosomes may appear in the peripheral blood during this time.

THE COMBINED IN-VITRO-VIVO METHOD

In this method equal parts of varying dilutions of the chemical under study are mixed with blood trypanosome emulsion and kept at 37-40 C., when the whole or a part of each is injected intraperi-

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[4] Jour. Infect. Dis., 1915, 17, p. 79.

TABLE 4

THE IN-VITRO-VIVO CENTRIFUGE METHOD (TRYPANOCIDAL ACTIVITY OF ARSENOBENZOL FOR T. EQUIPERDUM)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 320,000,000.
Approximate number of trypanosomes in 1 c.c. of diluted blood: 106,000,000.
Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.
Temperature of medication: 40 C.
Duration of exposure: 15 minutes + centrifugation.
Approximate amount of chemical in contact with the trypanosomes: given in the table.
Approximate number of trypanosomes injected: 53,000,000.

Rat	Weight, gm.	Dilutions	Amount, gm.	Results of Daily Examinations of Tail Blood											
				1	2	3	4	5	6	7	8	9	10	11	12
1	90	1:1000	.001	—	—	—	—	—	—	—	—	—	—	—	—
2	83	1:2000	.0005	—	—	—	—	—	—	—	—	D			
3	84	1:4000	.00025	—	—	—	—	—	—	—	—	—	—	—	—
4	72	1:8000	.000125	—	—	—	—	—	—	—	—	—	—	D	
5	80	Control	0	Few	Few	Few	+	++	++	D					
6	91	Control	0	—	—	—	Few	+++	D						
7	97	Control	0	—	Few	Few	+	+	+	++	++	D			
8	82	Control	0	—	Few	+	+++	++++	++++	D					

toneally into white rats to determine the degree of trypanocidal activity. As a part of the drug is injected, the action of the drug is both in vitro and in vivo and due care must be exercised against the administration of lethal doses of the drug.

Technic.—A series of dilutions of the drug under study is prepared in sterile normal salt or Ringer's solution in small sterile test tubes (1 × 6 cm.) and in amounts of 0.5 c.c. The series is placed in a water bath at 37-40 C. to be warmed to body temperature.

A rat heavily infected with T. equiperdum or T. brucei is selected, and a count made of the number of trypanosomes per cubic centimeter of blood.

Blood is then secured from the heart or vessels of the neck in sufficient sterile 1% solution of sodium citrate in normal salt or Ringer's solution warmed to 40 C. to give the desired dilution. Each cubic centimeter of this suspension should contain at least 20,000,000 trypanosomes. After a count has been made and the amount of dilution calculated, it is good practice to place the diluent in a small graduated cylinder and bleed the rat from the neck into the cylinder by means of a sterile funnel until the desired amount of blood has been added. This mixture is then gently shaken and afterward kept at a temperature of 37-40 C. in a water bath.

At an appointed time 0.5 c.c. of the trypanosome emulsion is added to each tube containing the solution of chemical and to a series of controls containing 0.5 c.c. of sterile normal salt or Ringer's solution. Each tube is gently shaken and then kept in the water bath at 37-40 C. for 15 minutes. The trypanosomes should be added to the chemical at intervals of one-half minute, which permits sufficient time for the injection of each.

At the end of the period of exposure 0.5 c.c. of the blood trypanosome chemical mixture is injected into the peritoneum of a rat. One-half minute is assigned for each injection. A 1-c.c. Record syringe fitted with a 22 platinum iridium needle is well adapted for the injections. After each injection there is sufficient time to wash out the syringe and needle several times with hot normal salt solution conveniently placed near the operator.

TABLE 5

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF BICHLORID OF MERCURY AND ARSENOBENZOL FOR T. EQUIPERDUM)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 240,000,000.
Dilution of blood contains approximately per cubic centimeter: 120,000,000.
Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.
Temperature of medication: 39 C.
Duration of exposure: 15 minutes.
Amount of chemical trypanosome mixture injected: 0.5 c.c.
Approximate number of trypanosomes injected: 30,000,000.
Approximate amount of chemical injected: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Bichlorid Hg	1	70	1 : 5000	.0002
	2	75	1 : 10,000	.0001
	3	122	1 : 15,000	.000066
	4	130	1 : 20,000	.00005
	5	105	1 : 30,000	.000033
	6	107	1 : 40,000	.000025
	7	68	1 : 80,000	.0000125
Arsenobenzol	8	116	1 : 200	.005
	9	90	1 : 400	.0025
	10	106	1 : 800	.00125
	11	100	1 : 1600	.00062
	12	114	1 : 3200	.00031
	13	89	1 : 6400	.00015
	14	80	1 : 12,800	.000075
Controls	15	107	0	0
	16	92	0	0
	17	94	0	0
	18	90	0	0

Each rat is then kept under observation for at least 2 weeks and the blood examined daily for trypanosomes. In this manner the degree of trypanocidal activity may be determined according to the time when trypanosomes appear and the duration of life as compared with that of the controls.

The results of a few of the series of experiments conducted with various substances are shown in Tables 5 to 8.

The viability of the trypanosomes under the conditions of this technic is indicated in Table 9, which gives the results of one of several experiments to determine this point. The uniform success with which the controls infect rats also indicates that T. equiperdum withstands very well an exposure in normal salt solution at 37 to 40 C. for at least 15 minutes. As shown in these and other experiments, the 15-minute period of exposure does not materially lower the viability or virulence of these trypanosomes.

As a part of the chemical is injected into the peritoneum of the rat, its trypanocidal activity is probably continued in vivo. To test the degree of trypanocidal activity under these conditions, a number of experiments were conducted with the same solutions of chemical and trypanosome emulsions, in some of which the two emulsions

TABLE 5—*Continued*

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF BICHLORID OF MERCURY AND ARSENOBENZOL FOR T. EQUIPERDUM)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 240,000,000.
Dilution of blood contains approximately per cubic centimeter: 120,000,000.
Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.
Temperature of medication: 39 C.
Duration of exposure: 15 minutes.
Amount of chemical trypanosome mixture injected: 0.5 c.c.
Approximate number of trypanosomes injected: 30,000,000.
Approximate amount of chemical injected: stated in the table.

Results of Daily Examinations of Tail Blood													
1	2	3	4	5	6	7	8	9	10	11	12	13	14
—	—	—	D										
—	—	—	—	—	—	—	—	—	—	—	—	D	
—	—	—	—	—	—	Few	Few	+	++++	D			
—	—	—	—	—	—	—	—	—	—	—	—	D	
—	—	—	—	—	—	Few	Few	+	++	++	+++	+++	D
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	D			
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	D				
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	+	+++	++++	D									
—	+	++	++++	D									
Few	+	+++	++++	D									
Few	+	+++	D										

were mixed in the usual manner and injected, and in others of which the emulsions were injected in the same amounts but separately. The results of two such experiments are shown in Tables 10 and 11.

As shown in Table 10 the mercurials, in doses which are barely sublethal by intraperitoneal injection, exert some trypanocidal activity when injected intraperitoneally at the same time as the trypanosomes. When these are mixed in the same amounts in vitro for 15 minutes and injected, a greater trypanocidal activity is apparent.

With arsenobenzol, however, the differences are not so apparent because of the very high trypanocidal activity of this substance (Table 11). With higher dilutions than those shown in Table 11 the influence of a 15-minute exposure in vitro in increasing trypanocidal activity becomes apparent.

RESULTS WITH THE COMBINED IN-VITRO-VIVO METHOD

We have made comparative tests with our routine in-vivo and combined in-vitro-vivo methods, of a number of substances, with interesting results.

TABLE 6

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF BICHLORID OF MERCURY AND ARSENOBENZOL FOR T. EQUIPERDUM)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 312,000,000.
Dilution of blood contains approximately per cubic centimeter: 104,000,000.
Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.
Temperature of medication: 38 C.
Duration of exposure: 15 minutes.
Amount of chemical trypanosome mixture injected: 0.5 c.c.
Approximate number of trypanosomes injected: 26,000,000.
Approximate amount of chemical injected: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Bichlorid of mercury	1	100	1 : 10,000	.0001
	2	102	1 : 15,000	.000066
	3	110	1 : 20,000	.00005
	4	115	1 : 30,000	.000033
	5	67	1 : 40,000	.000025
	6	94	1 : 60,000	.000016
	7	89	1 : 80,000	.0000125
	8	85	1 : 120,000	.0000083
	9	90	1 : 160,000	.0000062
	10	102	1 : 240,000	.00000416
Arsenobenzol	11	91	1 : 400	.0025
	12	80	1 : 600	.0015
	13	55	1 : 800	.00125
	14	71	1 : 1200	.00083
	15	113	1 : 1600	.00062
	16	89	1 : 2400	.00041
	17	84	1 : 4800	.000208
	18	105	1 : 9600	.000104
	19	91	1 : 19,200	.000052
	20	88	1 : 40,000	.000026
Control	21	87	0	0
	22	93	0	0
	23	77	0	0

TABLE 7

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF VARIOUS MERCURIALS AND PHENOL FOR T. EQUIPERDUM)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 256,000,000.
Dilution of blood contains approximately per cubic centimeter: 64,000,000.
Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.
Temperature of medication: 40 C.
Duration of exposure: 15 minutes.
Amount of chemical trypanosome mixture injected: 0.5 c.c.
Approximate number of trypanosomes injected: 16,000,000.
Approximate amount of chemical injected: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Bichlorid Hg	1	105	1 : 2500	.0004
	2	160	1 : 5000	.0002
	3	102	1 : 10,000	.0001
	4	128	1 : 20,000	.00005
	5	100	1 : 40,000	.000025
Salicylate Hg	6	132	1 : 2500	.0004
	7	102	1 : 5000	.0002
	8	85	1 : 10,000	.0001
	9	94	1 : 20,000	.00005
	10	103	1 : 40,000	.000025
Calomel	11	100	1 : 2500	.0004
	12	82	1 : 5000	.0002
	13	85	1 : 10,000	.0001
	14	89	1 : 20,000	.00005
	15	76	1 : 40,000	.000025
Phenol	16	100	1 : 100	.01
	17	87	1 : 200	.005
	18	77	1 : 400	.0025
	19	87	1 : 800	.00125
	20	93	1 : 1600	.000625
Control	21	76	0	0
	22	70	0	0
	23	58	0	0
	24	78	0	0

TABLE 6—*Continued*

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF BICHLORID OF MERCURY AND ARSENOBENZOL FOR T. EQUIPERDUM)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 312,000,000.

Dilution of blood contains approximately per cubic centimeter: 104,000,000.
Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.
Temperature of medication: 38 C.
Duration of exposure: 15 minutes.
Amount of chemical trypanosome mixture injected: 0.5 c.c.
Approximate number of trypanosomes injected: 26,000,000.
Approximate amount of chemical injected: stated in the table.

Results of Daily Examinations of Tail Blood

1	2	3	4	5	6	7	8	9	10	11	12	13	14
—	—	—	—	D									
—	—	—	D										
—	—	—	—	Few	++	+++	D						
—	—	—	—	Few	+++	D							
—	—	—	Few	+++	++++	++++	++++	D					
—	—	—	Few	++	D								
—	—	Few	+	D									
—	—	Few	+	+++	D								
—	+	++	+++	++++	D								
—	—	Few	+	+++	D								
—	—	—	—	—	—	—	—	—	—	D			
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	D							
—	—	—	—	—	—	—	D						
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	D							
Few	++	++++	D										
—	—	Few	++	D									
Few	+	++	+++	D									

TABLE 7—*Continued*

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF VARIOUS MERCURIALS AND PHENOL FOR T. EQUIPERDUM)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 256,000,000.

Dilution of blood contains approximately per cubic centimeter: 64,000,000.
Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.
Temperature of medication: 40 C.
Duration of exposure: 15 minutes.
Amount of chemical trypanosome mixture injected: 0.5 c.c.
Approximate number of trypanosomes injected: 16,000,000.
Approximate amount of chemical injected: stated in the table.

Results of Daily Examinations of Tail Blood

1	2	3	4	5	6	7	8	9	10	11	12
—	—	—	D								
—	—	—	—	—	D						
—	—	—	—	Few	++	+++	++++	D			
—	—	—	—	—	—	—	—	Few	Few	—	+++
—	—	—	—	—	Few	+	++++	D			
—	—	—	D								
—	—	—	—	D							
—	D										
—	—	—	—	—	—	D					
—	—	—	—	—	—	—	—	—	—	—	Few
—	Few	Few	D								
—	—	+++	D								
Few	+	+++	D								
Few	+	+++	D								
Few	+	+++	D								
—	—	—	—	D							
—	—	—	—	—	—	—	—	+	+++	++++	D
—	—	—	—	D							
—	—	Few	+++	++++	D						
Few	+	+++	++++	++++	D						
Few	+	++++	D								
Few	+	++++	D								
Few	+	++++	D								
Few	+	++++	D								

TABLE 8

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF VARIOUS NEW COMPOUNDS OF MERCURY FOR T. EQUIPERDUM)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 192,000,000.
Dilution of blood contains approximately per cubic centimeter: 48,000,000.
Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.
Temperature of medication: 37 C.
Duration of exposure: 15 minutes.
Amount of chemical trypanosome mixture injected: 0.5 c.c.
Approximate number of trypanosomes injected: 12,000,000.
Approximate amount of chemical injected: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Bichlorid	1	78	1 : 2500	.0004
	2	70	1 : 5000	.0002
	3	95	1 : 10,000	.0001
	4	95	1 : 20,000	.00005
	5	108	1 : 40,000	.000025
No. 41	6	78	1 : 2500	.0004
	7	92	1 : 5000	.0002
	8	80	1 : 10,000	.0001
	9	97	1 : 20,000	.00005
	10	87	1 : 40,000	.000025
No. 2	11	101	1 : 2500	.0004
	12	103	1 : 5000	.0002
	13	85	1 : 10,000	.0001
	14	89	1 : 20,000	.00005
	15	90	1 : 40,000	.000025
No. 4	16	78	1 : 2500	.0004
	17	92	1 : 5000	.0002
	18	82	1 : 10,000	.0001
	19	115	1 : 20,000	.00005
	20	88	1 : 40,000	.000025
	21	83	0	0
	22	90	0	0
	23	70	0	0
	24	80	0	0

TABLE 9

THE VIABILITY OF T. EQUIPERDUM IN THE COMBINED IN-VITRO-VIVO METHOD

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 368,000,000.
Dilution of blood contains approximately per cubic centimeter: 92,000,000.
Temperature of medication: 37 C.
Duration of exposure: stated in the table.
Amount of normal-salt-solution trypanosome mixture injected: 0.5 c.c.
Approximate number of trypanosomes injected: 23,000,000.

Rat	Weight, gm.	Exposure
1	110	At once
2	100	15 min.
3	62	30 min.
4	102	45 min.
5	99	60 min.
6	106	1½ hr.
7	100	2 hr.

TABLE 8—*Continued*

THE COMBINED IN-VITRO-VIVO METHOD (TRYPANOCIDAL ACTIVITY OF VARIOUS NEW COMPOUNDS OF MERCURY FOR T. EQUIPERDUM)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 192,000,000.
Dilution of blood contains approximately per cubic centimeter: 48,000,000.
Proportion of solution of chemical to trypanosome emulsion: 0.5 c.c. + 0.5 c.c.
Temperature of medication: 37 C.
Duration of exposure: 15 minutes.
Amount of chemical trypanosome mixture injected: 0.5 c.c.
Approximate number of trypanosomes injected: 12,000,000.
Approximate amount of chemical injected: stated in the table.

Results of Daily Examinations of Tail Blood

1	2	3	4	5	6	7	8	9	10	11	12
—	—	—	D								
—	—	—	—	—	—	Few	+	+++	++++	++++	D
—	—	—	—	—	—	Few	+	++	++++	D	
—	—	—	—	Few	+	+	+++	++++	++++	D	
—	—	Few	Few	+	+	++	++++	D			
—	—	—	—	—	—	—	—	Few	++	++	D
—	—	—	—	—	—	—	Few	+	+++	+++	D
—	—	—	—	D							
—	—	—	—	Few	Few	+	+++	++++	++++	++++	++++
—	—	Few	+	+	++	++++	++++	++++	D		
—	—	—	—	—	—	—	—	—	—	D	
—	—	—	—	—	—	—	—	—	—	D	
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	Few	+	+++	++++	D					
—	—	—	—	—	—	—	D				
—	—	—	—	—	—	Few	Few	Few	+	+++	D
—	—	—	—	—	Few	+	+++	++++	D		
—	—	—	—	Few	+	++	+++	D			
—	—	Few	Few	+	++	++++	D				
—	—	Few	Few	+	++	++++	D				
—	—	Few	+	++++	++++	++++	++++	D			
—	—	Few	+	++++	++++	D					
—	—	Few	+	++++	++++	D					

TABLE 9—*Continued*

THE VIABILITY OF T. EQUIPERDUM IN THE COMBINED IN-VITRO-VIVO METHOD

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 368,000,000.
Dilution of blood contains approximately per cubic centimeter: 92,000,000.
Temperature of medication: 37 C.
Duration of exposure: stated in the table.
Amount of normal-salt-solution trypanosome mixture injected: 0.5 c.c.
Approximate number of trypanosomes injected: 23,000,000.

Results of Daily Examination of Tail Blood

1	2	3	4	5	6	7	8	9	10	11	12	13	14
Few	++	++++	++++	++++	D								
—	Few	+	+++	++++	++++	D							
—	—	—	—	—	Few	++	+++	++++	++++	D			
—	—	—	—	—	Few	++	+++	++++	++++	++++	D		
—	—	—	—	—	—	—	—	—	Few	+	+	++	+++
—	—	—	—	—	—	—	—	—	—	Few	Few	Few	D
—	—	—	—	—	—	—	—	—	—	—	—	—	—

TABLE 10

THE COMBINED IN-VITRO-VIVO METHOD COMPARED WITH AN IN-VITRO TEST (TRYPANOCIDAL ACTIVITY OF SALICYLATE OF MERCURY IN ALKALINE SOLUTION)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 320,000,000.

Dilution of blood contains approximately per cubic centimeter: 80,000,000.

Proportion of solution of chemical to trypanosome emulsion in the in-vitro-vivo test: 0.5 c.c. + 0.5 c.c.

Temperature of medication: 37 C.

Duration of exposure: 15 minutes.

Amount of chemical solution injected alone in the in-vivo test: 1 c.c.

Approximate number of trypanosomes injected in both tests: 20,000,000.

Approximate amount of chemical injected in both tests: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Mixed	1	90	1 : 2500	.0004
	2	103	1 : 5000	.0002
	3	95	1 : 10,000	.0001
	4	62	1 : 20,000	.00005
Separate	5	95	1 : 2500	.0004
	6	70	1 : 5000	.0002
	7	70	1 : 10,000	.0001
	8	82	1 : 20,000	.00005
Alkali	9	81	Undiluted	
	10	62	1 : 2	
35 Min.	11	80	Control	
	12	75	Control	
	13	79	Control	
	14	71	Control	

TABLE 11

THE COMBINED IN-VITRO-VIVO METHOD COMPARED WITH AN IN-VIVO TEST (TRYPANOCIDAL ACTIVITY OF ARSENOBENZOL)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 128,000,000.

Dilution of blood contains approximately per cubic centimeter: 32,000,000.

Proportion of solution of chemical to trypanosome emulsion in the in-vitro-vivo test: 0.5 c.c. + 0.5 c.c.

Temperature of medication: 38 C.

Duration of exposure: 15 minutes.

Amount of chemical solution injected alone in the in-vivo test: 0.5 c.c.

Amount of chemical trypanosome mixture injected in the vitro-vivo test: 1 c.c.

Approximate number of trypanosomes injected in both tests: 8,000,000.

Approximate amount of chemical injected in both tests: stated in the table.

	Rat	Weight, gm.	Dilutions	Amount, gm.
Mixed	1	102	1 : 2000	.0005
	2	101	1 : 4000	.00025
	3	120	1 : 8000	.000125
	4	102	1 : 16,000	.000062
Separate	5	105	1 : 2000	.0005
	6	96	1 : 4000	.00025
	7	135	1 : 8000	.000125
	8	136	1 : 16,000	.000062
	9	103	Control	0
	10	92	Control	0
	11	88	Control	0
	12	102	Control	

TABLE 10—*Continued*

THE COMBINED IN-VITRO-VIVO METHOD COMPARED WITH AN IN-VITRO TEST (TRYPANOCIDAL ACTIVITY OF SALICYLATE OF MERCURY IN ALKALINE SOLUTION)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 320,000,000.
Dilution of blood contains approximately per cubic centimeter: 80,000,000.
Proportion of solution of chemical to trypanosome emulsion in the in-vitro-vivo test: 0.5 c.c. + 0.5 c.c.
Temperature of medication: 37 C.
Duration of exposure: 15 minutes.
Amount of chemical solution injected alone in the in-vivo test: 1 c.c.
Approximate number of trypanosomes injected in both tests: 20,000,000.
Approximate amount of chemical injected in both tests: stated in the table.

Results of Daily Examinations of Tail Blood

1	2	3	4	5	6	7	8	9	10	11	12	13
Hemolyzed												
—	—	—	—	—	—	—	—	—	—	—	Few	D
—	—	—	—	—	—	—	—	—	—	—	Few	D
—	—	—	Few	Few	D							
—	—	—	—	—	—	—	D					
—	—	Few	+	+++	++++	D						
—	—	Few	+	++	++	D						
—	Few	+++	++++	++++	++++	D						
—	—	—	—	—	D							
—	—	—	—	—	—	—	—	—	—	—	Few	D
—	Few	+	++++	++++	D							
Few	Few	+	++++	++++	++++	D						
—	Few	+	+	+	D							
Few	Few	+	+++	++++	++++	D						

TABLE 11—*Continued*

THE COMBINED IN-VITRO-VIVO METHOD COMPARED WITH AN IN-VIVO TEST (TRYPANOCIDAL ACTIVITY OF ARSENOBENZOL)

Approximate number of trypanosomes in seed rat per cubic centimeter of blood: 128,000,000.
Dilution of blood contains approximately per cubic centimeter: 32,000,000.
Proportion of solution of chemical to trypanosome emulsion in the in-vitro-vivo test: 0.5 c.c. + 0.5 c.c.
Temperature of medication: 38 C.
Duration of exposure: 15 minutes.
Amount of chemical solution injected alone in the in-vivo test: 0.5 c.c.
Amount of chemical trypanosome mixture injected in the vitro-vivo test: 1 c.c.

Approximate amount of chemical injected in both tests: stated in the table.

Results of Daily Examinations of Tail Blood

1	2	3	4	5	6	7	8	9	10	11	12
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	Few	++	++++	++++	D	
—	—	—	—	—	Few	+++	++++	++++	++++	D	
—	—	—	—	—	—	—	++	++++	++++	++++	D
—	—	—	—	—	—	—	—	+	+++	++++	D

TABLE 12

Comparison of the Trypanocidal Activity of Arsenobenzol in the In-Vivo and the Combined In-Vitro-Vivo Methods

Method	Rat	Weight, gm.	Dose
Routine In-Vivo	1*	116	.004†
	2	82	.003
	3	83	.002
	4	102	.001
	5	110	Control
	6	122	Control
Combined In-Vitro-Vivo	7	80‡	.00164§
	8	71	.00082
	9	89	.00041
	10	84	.000208
	11	105	.000104
	12	91	.000052
	13	88	.000026
	14	85	.000013
	15	87	Control
	16	93	Control

* Rats infected by intraperitoneal injection of 500,000 T. equiperdum. This is a greater infection than usual in our routine tests and with a resistant strain of T. equiperdum—facts which account for the failure of the 0.002-gram dose of arsenobenzol to sterilize.[4]

[4] Kolmer, Schamberg and Raiziss: Jour. Infect. Dis., 1917, 20, p. 35.

TABLE 13

Comparison of the Trypanocidal Activity of Compound No. 85-15 (a Derivative of Arsenbenzol) in the In-Vivo and Combined In-Vitro-Vivo Methods

Method	Rat	Weight, gm.	Dose		
				1	2
Routine In-Vivo	1*	87	.008†	—	—
	2	144	.007	—	—
	3	122	.006	—	—
	4	102	.004	—	—
	5	86	.002	—	+
	6	110	Control	—	+
	7	122	Control	—	+
Combined In-Vitro-Vivo	8	80‡	.01§	—	—
	9	95	.005	—	—
	10	100	.0025	—	—
	11	110	.00125	—	—
	12	90	Control	—	—
	13	65	Control	Few	+
	14	70	Control	—	—

* Rats infected by intraperitoneal injection with 500,000 T. equiperdum.

† Dose of Compound No. 85-15 per 100 gm. of body weight; injected intravenously 24 hours after infection.

TABLE 14

Comparison of the Trypanocidal Activity of Bichlorid of Mercury in the In-Vivo and the Combined In-Vitro-Vivo Methods

Method	Rat	Weight, gm.	Dose
Routine In-Vivo	1*	49	.0003†
	2	66	.0002
	3	55	.0001
	4	70	.00005
	5	80	Control
	6	65	Control
Combined In-Vitro-Vivo	7‡	78	.0004§
	8	70	.0002
	9	95	.0001
	10	95	.00005
	11	108	.000025
	12	90	Control
	13	70	Control

* Rats infected by intraperitoneal injection with 120,000 T. equiperdum 24 hours before the intravenous administration of bichlorid of mercury.

† Doses of bichlorid of mercury per 100 gm. of body weight.

TABLE 12—*Continued*

COMPARISON OF THE TRYPANOCIDAL ACTIVITY OF ARSENOBENZOL IN THE IN-VIVO AND THE COMBINED IN-VITRO-VIVO METHODS

Results of Daily Examinations of Tail Blood														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	Few	++	++++	D		
—	—	—	—	—	—	—	—	Few	++	+++	++++	++++	++++	D
—	+	+++	++++	++++	++++	D								
—	+	+++	++++	++++	D									
—	—	—	—	—	—	—	—	—	—	D				
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	D								
—	—	—	—	—	—	—	D							
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	D								
—	--	—	—	—	—	—	—	—	—	Few	+	++	++++	D
Few	++	++	++++	D										
—	+	++	++++	D										

† Dose of arsenobenzol per 100 gm. of body weight; injected intravenously 24 hours after injection.

‡ Combined in-vitro-vivo method conducted with 26,000,000 trypanosomes.

§ Amount of arsenobenzol to which the trypanosomes had been exposed for 15 minutes in vitro.

TABLE 13—*Continued*

COMPARISON OF THE TRYPANOCIDAL ACTIVITY OF COMPOUND NO. 85-15 (A DERIVATIVE OF ARSENOBENZOL) IN THE IN-VIVO AND COMBINED IN-VITRO-VIVO METHODS

Results of Daily Examinations of Tail Blood												
3	4	5	6	7	8	9	10	11	12	13	14	15
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	Few	++++	++++	++++	++++	++++	++++
+++	++++	++++	D									
+++	++++	++++	++++	D								
+++	++++	++++	D									
—	—	—	—	—	—	—	—	D				
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	Few	+	++	+++	++++	++++	D				
+++	++++	D										
—	—	Few	+	++	++	++++	D					

‡ Combined in-vitro-vivo method conducted with 42,000,000 T. equiperdum.

§ Amount of Compound No. 85-15 to which the trypanosomes had been exposed for 15 minutes in vitro.

TABLE 14—*Continued*

COMPARISON OF THE TRYPANOCIDAL ACTIVITY OF BICHLORID OF MERCURY IN THE IN-VIVO AND THE COMBINED IN-VITRO-VIVO METHODS

Results of Daily Examinations of Tail Blood														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
+	++++	++++	D											
+	++	++++	++++	++++	D									
+	+	+++	++++	D										
+	++	++++	D											
+	+++	++++	D											
+	+++	++++	D											
—	—	—	D¶											
—	—	—	—	—	—	Few	+	+++	++++	++++	D			
—	—	—	—	—	—	Few	+	++	++++	D				
—	—	—	—	Few	+	+	+++	++++	D					
—	—	Few	Few	+	+	++	++++	D						
—	—	Few	+	++++	++++	++++	D							
—	—	Few	+	++++	++++	D								

‡ Combined in-vitro-vivo method conducted with 12,000,000 T. equiperdum.

§ Amount of bichlorid of mercury to which the trypanosomes had been exposed for 15 minutes in vitro.

¶ Death probably due to toxicity of mercury.

As previously stated, the routine in-vivo test is conducted by infecting rats with intraperitoneal injections of T. equiperdum, averaging 120,000 trypanosomes per animal. Twenty-four hours later the drug is administered intravenously according to the body weight of each rat, each dose being dissolved in 1 c.c. of sterile distilled water.

The combined in-vitro-vivo tests were conducted by exposing about 25,000,000 to 50,000,000 T. equiperdum contained in 0.5 c.c. diluted blood to varying amounts of the chemical dissolved in 0.5 c.c. of normal salt solution.

The results observed with both methods with several substances are shown in Tables 12, 13, and 14 as examples of the results of these tests.

In Table 15 is given a summary of the results obtained with both tests with a number of new compounds prepared in the course of our chemotherapeutic researches, and several well-known bactericides.

TABLE 15

COMPARATIVE RESULTS WITH THE ROUTINE IN-VIVO AND THE COMBINED IN-VITRO-VIVO METHODS

Substance*	Routine In-Vivo Test		Combined In-Vitro-Vivo Test	
	Dose per 100 gm. Body Weight	Results (over period of 15 days)	Dose	Results (over period of 15 days)
Arsenobenzol	.0015	Trypanocidal	.000052	Trypanocidal
85-15 (a derivative of arsenobenzol)	.006	Trypanocidal	.00125	Trypanocidal
39 (a mercurial compound of naphthaline series)	.0003†	Not trypanocidal	.00025	Trypanocidal
42 (a compound of guaiacol-mercury)	.0004†	Not trypanocidal	.00025	Trypanocidal
47 (a derivative of guaiacol)	.002†	Not trypanocidal	.001	Trypanocidal
60 (an organic compound of copper)	.0009†	Not trypanocidal	.0005	Trypanocidal
64 (copper salicylate)	.001†	Not trypanocidal	.001	Trypanocidal
Copper sulfate	.001†	Not trypanocidal	.0025	Trypanocidal
Phenol	.01†	Not trypanocidal	.005	Trypanocidal
Bichlorid of mercury	.0003†	Not trypanocidal	.000033	Inhibitory for 4 days
2 (a mercury compound of the naphthol series)	.0003†	Not trypanocidal	.00005	Trypanocidal
41 (a mercury compound)	.0002†	Not trypanocidal	.0004	Inhibitory for 8 days

* The chemistry of these new compounds will be given in a later communication.
† About the lethal dose for rats by intravenous injection per 100 gm. of body weight.

As shown in the tables the results obtained with the combined in-vitro-vivo method are generally clear-cut and decisive.

At times the animals are injected intraperitoneally with a lethal dose of the drug; in conducting the combined in-vitro-vivo method these doses are to be avoided.

As shown in Table 12 arsenobenzol, or salvarsan, possesses a very high degree of trypanocidal activity in the combined in-vitro-vivo method. As shown in Tables 3, 4, 5, and 6 this activity is certainly, in part at least, due to the influence of arsenobenzol upon trypanosomes in vitro.

The combined in-vitro-vivo method always indicates a smaller trypanocidal dose for a compound than the routine in-vivo test.

Substances which in amounts just sublethal, exert no appreciable influence upon trypanosomes in vivo, in the combined in-vitro-vivo method either are definitely trypanocidal or in a certain dosage inhibit the multiplication of trypanosomes and their appearance in the peripheral blood for a certain number of days. In this manner results with the combined in-vitro-vivo method frequently yield information of value and serve as a guide for further researches with a particular and promising therapeutic compound or group of compounds.

CONCLUSIONS

Trypanocidal tests in vitro have been found of distinct value in chemotherapeutic researches in experimental trypanosomiasis.

Substances exerting a profound trypanocidal activity in vitro are likely to prove trypanocidal in vivo, provided the drug is sufficiently nontoxic to be administered in adequate dosage.

With the combined in-vitro-vivo method described herein, it has been found possible to detect the trypanocidal activity of new compounds which were without effect in vivo in amounts but slightly less than the sublethal dose.

By the methods described herein arsenobenzol, or salvarsan, has been shown to possess a high trypanocidal activity in vitro.

In-vitro methods have also demonstrated a trypanocidal activity on the part of mercurials which is not apparent in the in-vivo tests.